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Building on previous work where fluorescence spectroscopy has been used to detect sewage in rivers, a portable LED spectrophotometer was used for the first time to establish bacterial numbers in a range of water samples. A mixed-method approach was used with standard bacteria enumeration techniques on diluted river water and sewage works final effluent using a number of diluents (Ringer's solution, tap water and potable spring water). Fluorescence from uncultured dilutions was detected at a 280 nm excitation/360 nm emission wavelength (corresponding to the region of tryptophan and indole fluorescence) and compared with bacteria numbers on the same cultured sample. Good correlations were obtained for total coliforms, *E. coli* and heterotrophic bacteria with the portable LED spectrophotometer ($R^2 = 0.78, 0.72$ and 0.81 respectively). The results indicate that the portable spectrophotometer could be applied to establish the quality of drinking water in areas of poor sanitation that are subject to faecal contamination, where infrastructure failure has occurred in the supply of clean drinking water. This would be particularly useful where laboratory facilities are not at hand.

Keywords: portable fluorescence; drinking water quality; coliforms; *E. coli*; tryptophan; faecal contamination

Introduction

Drinking water containing pathogenic enteric organisms through faecal contamination is a worldwide problem. Affected water sources (streams, wells, boreholes, etc.) in developing countries can be responsible for ill health and higher mortality, particularly through diarrhoeal infections, affecting economic and social welfare [1–3]. The United Nations Millennium Development Goals aim to halve the number of people without access to clean drinking water by 2015, and the significance of this in improving access to clean water cannot be over-emphasized [4]. However, the latest World Health Organization (WHO) report highlights that these targets are not on track, with 2.6 billion and 884 million people, globally, lacking access to improved sanitation and drinking water respectively, particularly in sub-Saharan Africa and Asia [5]. Newly installed clean water supplies in developing countries may fail for a number of reasons, e.g. poor reliability and damage to infrastructure [6–8], with breakdown rates as high as 77% [1]. A few days of interrupted supply of clean drinking water causes drinking water to be sufficiently impaired so as to destroy the health benefit from the initial provision [7] because of high rates of bacterial survival as biofilms in taps, pipes and storage vessels [9–10]. For this reason, regular reassessment of water quality is required, to provide early warning signals of failing supplies [11]. This can lead to improved drinking water, which in turn benefits hygiene, education and local economic development [12]. New and improved technologies are needed to monitor water quality and supply, and would also be hugely beneficial in situations of humanitarian disaster [13]. The WHO therefore recommends the use of simple, more frequent detection methods, rather than less frequent, more complex methods to monitor and detect faecal contamination in drinking water [14].

Current detection methods of waterborne organisms are expensive, time-consuming (18–30 hours) and require skilled operators working in laboratory conditions, with virus detection [15] or genetic identification techniques [16] taking up to several weeks. Traditional standard methods used to test for the presence of heterotrophic bacteria (HB), total coliforms (TC), faecal coliforms (FC) and *Escherichia coli* (*E. coli*) include, inter alia, the multiple tube method (presence of gas and acid at 44 °C), membrane filtration (MF), and colony-forming units (cfu) on agar media [17]. The Colilert-18 assay system is based on a defined substrate medium containing 4-methylumbelliferyl-β-D-glucuronide (MUG) and ortho-nitrophenyl-β-D-galactopyranoside (ONPG), and

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can detect both TC and *E. coli* in water samples as one test [18]. As all pathogens cannot practically be assessed, indicator organisms are often used, as a surrogate for the presence of more sinister organisms such as typhoid and cholera. The group *Enterobacteriaceae* include TC, FC and *E. coli*, and detection of TC in drinking water therefore may indicate failure in the treatment or distribution systems. Total coliforms are traditionally defined as Gram-negative, anaerobic, rod-shaped bacteria. They do not form spores, are able to ferment lactose to acid and gas within 48 h at $36 \pm 2\,^{\circ}C$, and test positive for β-galactosidase [15,17]. However, since some are capable of growth in the environment outside of the gastric tract, TCs are considered unreliable indicators of sewage contamination, whereas *E. coli* is preferred as it is more likely to be present with faecal contamination. Furthermore, *E. coli* is thermotolerant, able to produce indole from tryptophan, and possesses the enzyme β-glucuronidase [19–20].

The use of fluorescence spectrophotometry as a tool to assess water quality in surface and ground waters has been reported and reviewed widely [21–22], and its application in the monitoring of natural and anthropogenic carbon is gaining momentum in the field. The development of fluorescence-scanning instruments to produce excitation–emission matrices (EEMs) has resulted in the ability to fingerprint the fluorescent organic fraction in water samples [23–24] via the identification of fluorophores, which correlate with the dissolved organic carbon content in rivers according to [25]. One emerging application of fluorescence is the detection of faecal contamination of water sources through the signal for the amino acid tryptophan and related compounds, which is related to microbial activity, nominally called peak T [22–23]. The peak T fluorophore has been successfully used in rivers to detect sewage and fluorescence increase in cultured river samples with bench-top fluorometers [26–28]. Specifically, peak T can be subdivided into two separate peaks: T1 ($\lambda_{ex} = 230$ nm, $\lambda_{em} =$ 350–360 nm) and T2 ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 350$–360 nm). Portable xenon lamp fluorescence devices (e.g. SMF-2, Safe Training Systems) have shown potential for *in situ* monitoring. The SMF-2 peak T response has shown good correlation with biochemical oxygen demand in river water when compared with a standard bench top instrument, the Varian Cary Eclipse ($R^2 = 0.72$, SMF-2; $R^2 = 0.95$, Cary Eclipse) [29], and offers the advantage that on-site measurements reduce the chance of sample perturbation effects during storage and transportation [30]. Recent technological advancements of fluorescence mean that light-emitting diodes (LEDs) in the UV range, substituting for the xenon lamp, can operate at low power, allowing greater portability and instantaneous results with little specialist technical knowledge. The aim of this paper is to assess the suitability of such a portable LED fluorometer to highlight failures in the treatment and distribution of drinking water. By comparing bacteria numbers gained from standard methods with the peak T2 fluorescence signal from diluted river or effluent samples using different dilution matrices, this paper demonstrates for the first time the correlations between microbial counts and fluorescence using an LED-based portable device.

Experimental section

Sampling

River and effluent samples were collected in clean glass 1 L bottles, from four locations in the West Midlands, UK, in April and May 2010. The River Rea (OS grid reference 061824) and the Bourn Brook (OS grid reference 045833) are urban rivers that flow through Birmingham and are tributaries of the River Tame. The River Severn (OS grid reference 814705) is a major watercourse in the UK, which discharges into the Bristol Channel in the south-west of the country. The final effluent sample was taken from Kidderminster sewage treatment works (STW) (OS grid reference 822738) serving a population equivalent of 56,600. Sample locations were chosen in order to gain sample variation in terms of dissolved organic carbon and bacteria flora and number. Samples and sample dilutions were stored at $4\,^{\circ}C$ in the dark. To minimize any effects of sample change, bacteria culturing and fluorescence analysis were completed on all dilution levels within six hours. Fluorescence (measured using a standard bench-top spectrophotometer and an LED-based fluorometer) was measured separately on each day and for each dilution. Fluorescence was always performed on uncultured samples, and the same sample was cultured for bacteria enumeration (HB, *E. coli*, TC and presumptive coliforms, PC) to enable comparisons. Dilutions for the Colilert method were made at the same time with identical dilutions in separate glass bottles.

Preparation of glassware and samples

Sterilization of media, glassware and diluents was achieved by autoclaving at $120\,^{\circ}C$ for 15 minutes. Glassware used for fluorescence samples was cleaned by acid-washing in 10% hydrochloric acid and rinsed in deionized (DI) (non-fluorescent) water to ensure no acid traces remained. Microbial work was performed under clean conditions near a flame on alcohol-swabbed benches. Three diluents: quarter-strength Ringer's solution (a salt solution used in microbiology for culturing purposes, made up with DI water), Birmingham tap water and potable spring water, Buxton, UK, were selected to provide a range of water types. Diluents were tested for their non-fluorescent properties both before and after autoclaving. For practicalities of bacteria counting and to test the fluorescence capabilities, sample dilutions of 10:1, 100:1, 1000:1 and 10,000:1 were prepared in 100 mL of sterilised diluent (glass bottle washed in 10% HCl, triple-rinsed in DI water). Possible contamination of the quartz cuvette was routinely checked by recording the fluorescence intensity of a DI blank at intervals throughout the sampling. The collected samples

were stored and used for three days while analysis with each diluent was performed in turn (i.e. day 1: Ringer's solution; day 2: tap water; day 3: spring water). The exception to this was the STW final effluent, where two separately collected samples were used to complete the dilution experiments. Three replications were made for each dilution, and blanks (from the diluent) were analysed in all cases. Undiluted samples were not cultured because of the high levels of bacteria present. Separate 100 mL dilutions were used for each replicate for Colilert-18 tests, and subsamples for the MF, HB and fluorescence analysis were taken from one sample bottle. Further dilutions of ×2, ×4, ×5, ×8 and ×20 were also made using the sterilised diluents (tap water, Ringer's solution, spring water) in glass vials (10% HCl acid-washed, triple-rinsed in DI water) for fluorescence analysis and comparison of the performance of two fluorescence instruments. Standard solutions of tryptophan and indole were prepared in pure water (18.2 mΩ).

Bacteria enumeration

Standard methods [17] were followed for enumeration of HB and PC by membrane filtration (PC/MF). Heterotrophic bacteria numbers were derived from 1 mL samples cultured with 20 mL of sterile non-selective agar (R2A, OXOID, CM 906) in sterile plastic Petri dishes. Colony-forming units were counted after incubating at 37 °C for 24 hours, where one colony was assumed to have been formed from one bacterium. Results are presented as cfu per 100 mL with all tests being performed in triplicate. The PC/MF numbers were determined by the MF method, whereby 10 mL of the sample was filtered through a sterile 0.45 μm cellulose nitrate filter (Millipore) and cultured on a sterile pad soaked in membrane lauryl sulphate broth (OXOID) and incubated at 35 °C for 24 hours. The presence and number of yellow colonies indicated the number of PC. All tests were performed in triplicate and results are presented as cfu per 100 mL. Total coliforms and *E. coli* were determined by the Colilert 18 (IDEXX) method [31], and prepared in clean, sterile, glass bottles. A hundred millilitres of the diluted sample was mixed with the manufacturer's reagent (a defined substrate medium containing MUG and ONPG), sealed in quanti-trays and incubated at 35 °C for 18 hours. The ONPG turns yellow when metabolized by coliforms, and MUG fluoresces under UV light when metabolized by *E. coli*. The MPN (most probable number) was determined by counting the number of wells that had turned yellow for TC and the number of fluorescent wells for *E. coli* (fluorescence bulb $\lambda_{ex} = 320$ nm). Counts were recorded via MPN tables (IDEXX) and results are presented as MPN per 100 mL.

Fluorescence

Fluorescence analysis was performed on a standard bench-top spectrophotometer (Cary Eclipse, Varian) and on a portable fluorimeter (SMF-4, Safe Training Systems, Wokingham, UK). The bench-top spectrophotometer was used to obtain EEMs. These were obtained from scans run at the following settings: photomultiplier tube voltage of 725 v, excitation and emission slit widths of 5 nm, excitation scans at 5 nm steps over the range 200 nm $< \lambda_{ex} <$ 400 nm, with emitted fluorescence detected at 300 nm $< \lambda_{em} <$ 500 nm. The fluorescence peak T1 and T2 intensity were recorded at $\lambda_{ex} = 230$ nm and 280 nm respectively, with emitted fluorescence recorded as the average over the wavelengths 358–362 nm. Regular blank scans were run with a sealed cell containing DI water. The same cell was used to determine the average intensity of the Raman line at $\lambda_{em} =$ 348 nm, using the same instrument settings as described above. All fluorescence intensities were corrected to a Raman peak intensity of 20 units [26].

Peak T2 fluorescence was also recorded on a portable fluorimeter (SMF-4, Safe Training Systems, Wokingham, UK), using an LED light source powered by four AA batteries with fixed $\lambda_{ex} = 280$ nm, $\lambda_{em} = 360$ nm; slit width of 10 nm ± 4 nm, centred on 360 nm. Data were automatically background subtracted by taking the sum of 16 readings (from binary multiples) without the LED excitation and subtracting it from the sum of 16 readings from the LED-excited sample. The displayed reading was taken as the sample reading. Sample analysis on both instruments was carried out using the same sample in a 1 cm pathway quartz cuvette (QG Helma). The cuvette containing the sample was analysed using one instrument followed by immediate reanalysis on the other. In all cases the samples were blank subtracted using the sterilised diluent blank. Tryptophan (VWR) and indole (Aldrich 99%) solutions were made to desired concentrations (0–100 μg L^{-1}) and used as test standards to examine instrument performance.

Results and discussion

The peak T2 intensities (measured at fixed wavelength pair $\lambda_{ex} = 280$ nm, $\lambda_{em} = 360$ nm), were determined for all samples and dilutions for both the SMF-4 and the Varian Cary Eclipse instruments (Figure 1). The data show an excellent correlation between the two instruments ($R^2 =$ 0.99) and indicate the two instruments' abilities to record the peak T2 intensity for raw samples and for a range of sample dilutions in different matrices. The background data for NO_3-N and PO_4 in the river samples and the estimated total bacteria counts determined from all tests is available in the supporting information, Table S1. In addition, Figure S1 shows the EEMs for the undiluted three river samples and one STW final effluent sample, and Table S2 provides the fluorescence intensities at Peaks T1, T2 and C (i.e. humic-like fluorescence with absorption in the visible range), as measured by the Varian Cary Eclipse instrument.

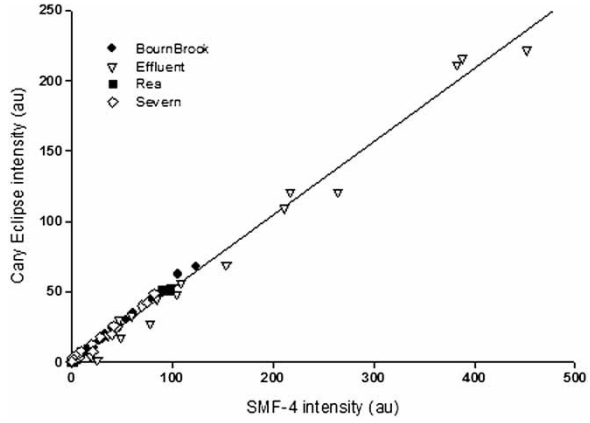

Figure 1. Comparison of fluorescence data measured by the two instruments for all samples. $\lambda_{ex} = 280$ nm, $\lambda_{em} = 360$ nm ($R^2 = 0.99$).

Fluorescence response to bacteria numbers

The peak T responses of both instruments to different bacteria numbers (per 100 mL) for each of the bacteria data sets (TC, *E. coli*, PC/MF and HB) are shown in Figure 2 and Table 1. The plots are presented on a log scale because dilution series were prepared by factors of 10. The variability observed in Figure 2 is principally due to the precision of the various count approaches used. The SMF-4 showed better correlations with bacteria numbers than the Varian Cary Eclipse instrument. The strongest correlations were found to be between the SMF-4 and HB ($R^2 = 0.81$) and between the SMF-4 and TC ($R^2 = 0.78$). The Varian Cary Eclipse instrument showed slightly weaker relationships than the SMF-4 throughout, and its strongest correlations were with *E. coli* ($R^2 = 0.65$) and HB ($R^2 = 0.72$). The variation in fluorescence at low MPN per 100 mL sample may be due to the presence of metals in the sewage, which quench the fluorescence or the precision of the methods used [32]. However, at the very high dilutions that were analysed in this work, this is considered unlikely. There is a clear difference in slopes between the two instruments when measuring the same samples. These differences are of the same order of magnitude as the differences observed when the instruments were used to measure the fluorescence intensities of indole and tryptophan standards (Figure S2), demonstrating that this is an effect of the optical design of the two instruments.

The bacteria numbers (cfu or MPN per 100 mL) obtained from each cultured bacteria group (HB, TC or *E. coli*), measured through the different enumeration tests, gave different peak T2 responses, as shown by the gradients in Figure 2. The group associated with the highest peak T2 fluorescence for the lowest number of bacteria was *E. coli*. This is evident by the change of intercept of the regression line for *E. coli* compared with TC for SMF-4 (Figure 2a and 2b). This can also be observed by comparing TC and HB, where TC data are more right-shifted than HB. Although HB gave a stronger correlation, more bacteria were required to give the same fluorescence intensity, indicating that some species present in HB contributed less fluorescence than others. The PC data obtained from MF lay between the TC and *E. coli*.

The results demonstrate that an increase in bacteria numbers can be correlated with an increase in fluorescence intensity with both the bench-top and the portable devices. There are a number of different compounds containing tryptophan residues that are associated with bacteria and biofilm exudates [33] that emit fluorescence in the peak T region. Tryptophan and indole are just two of these and are related to microbial and *E. coli* activity. *Escherichia coli* produces indole from lactose and tryptophan [20], and so the *E. coli* group of bacteria, if present, will have the highest fluorescence per number owing to its higher indole production. Both instruments gave strong correlations between *E. coli* and fluorescence intensity and between HB and fluorescence intensity. Interestingly, the SMF-4 results showed stronger correlations than the Cary Eclipse for all samples. *Escherichia coli* is the most important indicator species of faecal contamination, and its presence may indicate the occurrence of other pathogens such as cholera. Therefore, the ability to detect this group of bacteria demonstrates the potential of using peak T (and, specifically, peak T2), measured by a portable spectrophotometer, as a link to bacteria communities and, specifically, *E. coli*.

Comparison of bacteria enumeration methods

It is instructive to compare the bacteria enumeration methods used in this work because the Colilert-18 is an attractive method, being easily implementable in the field. The Colilert test results showed good repeatability between replicates of the same sample and with each diluent obtained, when comparing bacteria numbers. The TC result generally exceeded the *E. coli* result by a factor of 10 for the same Colilert-18 test ($R^2 = 0.754$). The HB counts were an order of magnitude higher than those of TC ($R^2 = 0.578$), indicating the presence of other bacteria species capable of growing at 37 °C. However, the Colilert method has been reported to give higher numbers than MF methods in some instances [34]. The MF test itself does not indicate definitively that these cultured bacteria are in fact all *E. coli*. Furthermore, it has been reported that the MF method can underestimate bacteria numbers possibly because of its inability to recover stressed bacteria, unlike the Colilert method [34]. It is plausible that coliforms (approximate size $0.5 \times 2\ \mu$m) may pass through the 0.45 μm membrane, demonstrating why higher numbers have been reported from the Colilert method. It is also possible that, due to bacteria clumping and attachment to colloidal or particulate material, bacteria numbers are a result of more than one bacterium per cfu, thus lowering the bacteria number per volume.

Detection limits

Sample fluorescence intensity repeatability for the SMF-4 was good and standard deviations (for more than three

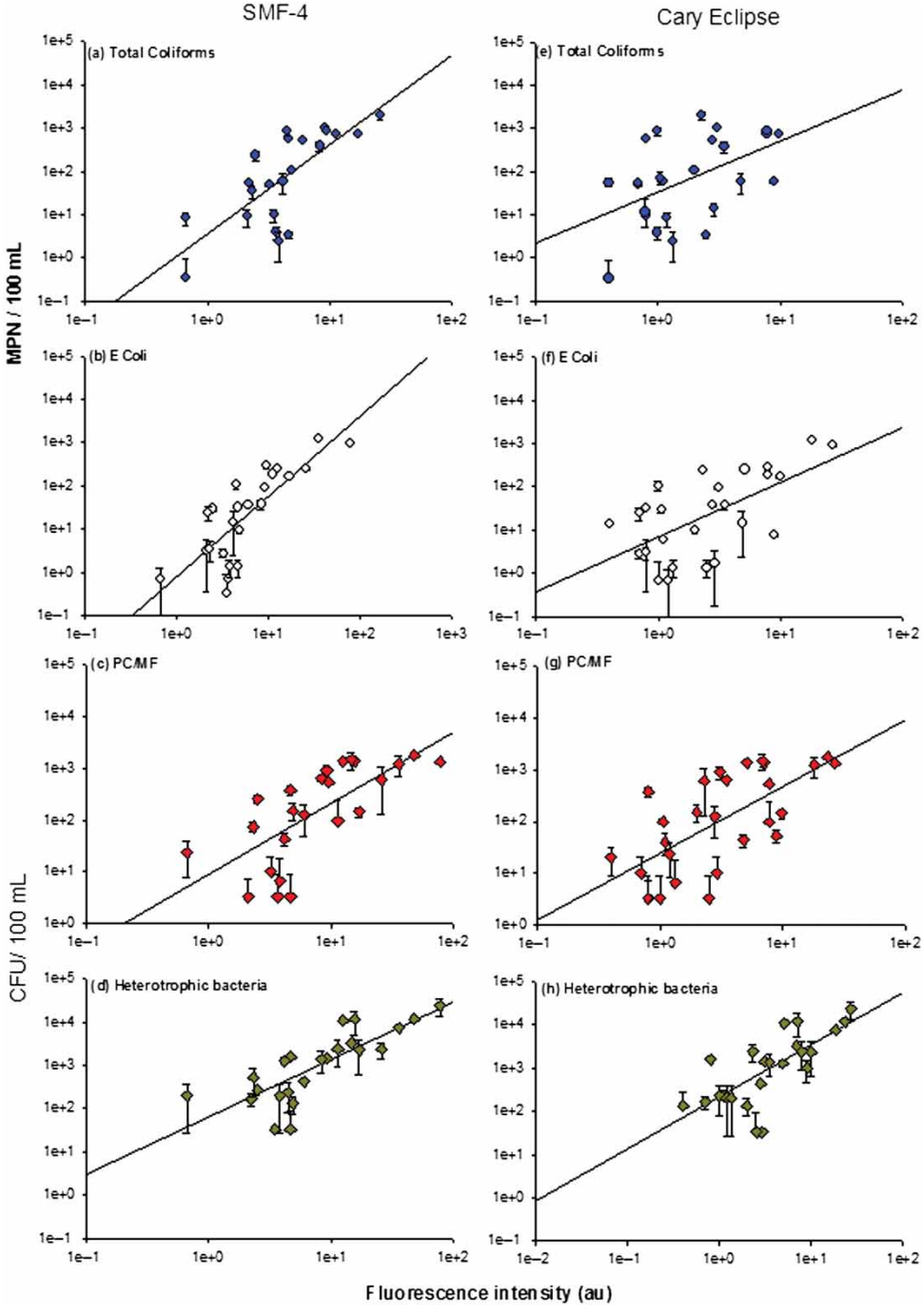

Figure 2. Fluorescence intensity (log scale) vs. bacterial enumeration (log scale) for SMF-4 (a–d) and Cary Eclipse (e–h). Plots (a) & (e): total coliforms via Colilert; (b) & (f): faecal coliforms via Colilert; (c) & (g): presumptive coliforms via MF; (d) & (h): heterotrophic bacteria on agar at 37 °C. Bacteria numbers are represented either as colony forming units (cfu/100 mL) or most probable number (MPN/100 mL).

measurements) were between 0 and 7. Standard deviations for the Cary Eclipse were between 0.18 and 4.5. Both instruments were able to detect the presence of a just a few bacteria per 100 mL. However false negative and false positive results meant that the instruments' detection limits at low bacteria and low fluorescence numbers (i.e. below 10 intensity units and 10 bacteria per 100 mL) could not be determined accurately. As a consequence, it was not straightforward to determine accurately the instruments' detection limits. However, limits of detection have been estimated from the data to be 10–100 MPN per 100 mL sample for *E. coli*, 500 cfu per 100 mL sample for HB and 200 MPN per 100 mL sample for TC. At dilutions of 10,000:1 and 1000:1, fluorescence detection was poor, but

Table 1. Gradient, intercept and R^2 linear correlation data for bacteria number and fluorescence for the SMF-4 fluorimeter and Cary Eclipse spectrophotometer.

	TC (MPN)	*E. coli* (MPN)	MF/PC (cfu)	HB (cfu)
SMF-4				
slope	69.08	22.92	25.49	325.48
intercept	−20.36	−33.16	92.59	−368.72
R^2	0.80	0.74	0.57	0.83
n	45	46	50	48
Cary Eclipse				
slope	85.34	46.11	57.22	650.96
intercept	98.27	−22.33	90.68	−63.19
R^2	0.24	0.75	0.59	0.69
n	45	46	50	48

Note: TC – total coliforms, MF/PC – presumptive coliforms, HB – heterotrophic bacteria.

in some cases bacteria could still be cultured from these dilutions.

The sample volume used for culturing is also a limitation for resolving detection limits. For example, the HB test has a detection limit of 100 bacteria per 100 mL as just 1 mL of sample is applied to the agar media for culturing. The use of replicates will, of course, increase the detection limit. Membrane filtration requires a 10 mL sample and so the detection limit is 10 per 100 mL, and the Colilert detection limit is 1 per 100 mL. Alternatively, through culturing, bacteria clumping may occur, which therefore will affect the detection limit. However, in fluorescence analysis, molecules that may be attached to the microbes will be observed via the optical window of the cuvette, thus clumping is not necessarily an issue; however, the sample volume is compromised (about 0.5 mL). Despite this, the results showed excellent agreements between bacteria numbers and fluorescence intensity.

Finally, interference from other fluorescing compounds may be a limit to detection. Polycyclic aromatic hydrocarbons (PAHs) have an intrinsic fluorescence close to the T1 region, and at high concentrations may impart a significant background in the T2 region. The intrinsic fluorescence of PAHS has been observed in landfill leachates and leachate- polluted rivers and groundwater. However, Tedetti *et al*. [35] found that tryptophan exhibited a higher sensitivity to fluorescence than PAHs (e.g. naphthalene) that might also generate peak T fluorescence. Therefore, where microbial communities are present, the tryptophan-like fluorescence would be expected to dominate over PAHs. Highly coloured, humic-rich water might likewise generate a background fluorescence in the peak T2 region from a fluorescence peak in the peak C region. However, our samples utilized river samples with typical organic carbon concentration and peak C fluorescence (Table S1, Figure S1), demonstrating the robustness of the fluorescence technique.

Conclusions

This study has built on previous research that used peak T fluorescence to detect contamination in water [30,36]. The peak T1 and T2 fluorescence data provided here reinforce the importance of this technique in detecting contamination. However, in the work reported here, by comparing the performance of a standard bench top spectrophotometer and a portable LED-based fluorimeter in the assessment of bacterial contamination of water, it has been shown that low levels of coliform and *E. coli* bacteria (less than 100 per 100 mL) can be detected by peak T2. Thus, the technique has the potential to provide a robust signal of bacterial contamination without the need for either expensive and time-consuming culture-independent measurements (e.g. qPCR) or culture-dependent assay methods (e.g. MF). As a result, the technique is simple enough to be used by expert and non-expert stakeholders alike. Further work is now required to apply the relationships found in the field to regions of poor sanitation and, in particular, to demonstrate the robustness of the relationships shown here, the ruggedness of the equipment in extreme conditions, and the reliability of the technique in predicting the safety of drinking water.

The application of an LED-based portable fluorescence device has the potential to achieve instantaneous results for the microbial load of potable water at modest cost and using minimal equipment and consumables, without waiting for results from sample culturing, thus potentially meeting the WHO requirements for more frequent, less sophisticated testing in the field rather than irregular and infrequent, but more comprehensive, tests.

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